

**DOCKET NO.: ALLE0047-101 (17006 CON2)**  
**U.S. Serial No. 09/845,512**

**PATENT**

### **REMARKS**

Upon entry of this response, claims 14, 16, 18, 19, 21 and 23-26 will be pending. New claims 25 and 26 are fully supported by the specification at, for example, paragraph bridging pages 5 and 6; and paragraph bridging pages 6 and 7. No new matter is added.

#### **The Claims Are Fully Described By The Specification**

Claims 14, 16, 18, 19, 21 and 23 are rejected under 35 U.S.C. 112, first paragraph, as allegedly lacking support by the specification, with respect to the terms “achieve a marked reduction [of a dystonia symptom]” or “substantially alleviate a [dystonia] symptom”. Applicant respectfully disagrees. However, for clarity, Applicant deleted the phrase “achieve a marked reduction [of a dystonia symptom]” from the claims.

The phrase “substantially alleviate a [dystonia] symptom” is fully supported by the specification. A “spasmodic torticollis” is also known as a dystonia (see the Specification, page 5, line 24). Example 2 (page 14) discloses that the symptoms of a spasmodic torticollis, or dystonia, include

spasmodic or tonic contractions of the neck musculature,  
producing stereotyped abnormal deviation of the head, the  
chin being rotated to one side, and the shoulder being  
elevated toward the side at which the head is rotated

Further, the specification discloses that these symptoms are “substantially alleviated” as the patient is “able to hold his head and shoulder in a normal position”. Thus, to “substantially alleviate” a dystonia symptom is, for example, to alleviate a symptom to a degree where the patient is able to hold his head and shoulder in a normal position.

Thus, the phrase “substantially alleviate a [dystonia] symptom” is fully supported by the specification.

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**The Claims Are Definite**

Claims 14, 16, 18, 19, 21 and 23 are rejected under 35 U.S.C. 112, second paragraph, as allegedly being indefinite, with respect to the terms “achieve a marked reduction [of a dystonia symptom]” or “substantially alleviate a [dystonia] symptom”. Applicant respectfully disagrees.

The phrase “achieve a marked reduction [of a dystonia symptom]” is deleted from the claims, rendering the rejection to this phrase moot.

The phrase “substantially alleviate a [dystonia] symptom” is definite with respect to the term “substantially”. As discussed above, the specification discloses that a dystonia symptom is “substantially” alleviated, for example, when a dystonia symptom is alleviated to a degree where the patient is able to hold his head and shoulder in a normal position. Thus, the claims are definite.

**The Claims Are Not Obvious**

Claims 14, 16, 18, 19, 21 and 23 are rejected under 35 U.S.C. 103(a) as allegedly being obvious over Ludlow et al. in view of Simpson et al. and Jankovic et al. Applicant respectfully disagrees. The claims are not obvious because:

- (I) 1 unit of botulinum toxin (BoNT) type F is not equivalent to 1 unit of BoNT-E, and**
- (II) The prior art teaches away from the use of BoNT-E after the use of BoNT-A**

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**(I) 1 Unit of Botulinum Toxin Type F Does Not Equal 1 Unit of Type E**

The Office Action alleges that Ludlow et al. teaches the dosage of BoNT-F, and admits that Ludlow et al. does not teach the dosage of BoNT-E. However, the Office Action alleges that it would have been obvious to one of ordinary skill to use the dosage of BoNT-F for that of BoNT-E, since both BoNT-F and BoNT-E block the release of acetylcholine (Office Action, page 6).

The Office Action's allegation is based on the premise that the dosage of BoNT-F is equivalent to that of BoNT-E. However, the Office Action has not shown that there is such an equivalency. In fact, it is well known that 1 unit of a BoNT has different biological efficacy from 1 unit of another BoNT of a different serotype. In fact, Ludlow et al. also teaches that 1 unit of BoNT of different serotypes do not have the same biological efficacy. For example, Ludlow et al. discloses that BoNT-A is about nine times more toxic than BoNT-F, despite the fact that both BoNT-A and BoNT-F block the release of acetylcholine (Ludlow et al, page 349, second column).

As BoNT-A and BoNT-F was known to have different biological efficacies, one of ordinary skill would also expect BoNT-E and BoNT-F to have different biological efficacies. Accordingly, one of ordinary skill would not use the same dosage of BoNT-F for that of BoNT-E to achieve the same therapeutic effects. Thus, it would not have been obvious for one of ordinary skill to use the claimed dosages for BoNT-E to treat a dystonia.

**(II) The prior art teaches away from the use of BoNT-E after the use of BoNT-A**

At the priority date of the present application, one of ordinary skill would expect that antibodies to BoNT-A would cross-react with BoNT-E, but not BoNT-F.

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For example, at the priority date of the present application, it was known that BoNT-A and -E cleave SNAP-25 (Schiavo et al., The Journal of Biological Chemistry 1993, 268(32):23784-23787, page 23784, enclosed herewith). As both BoNT-A and -E cleave SNAP-25, one of ordinary skill would expect the two enzymes to be physically similar. As they are physically similar, an antibody generated for one would cross-react with the other. Accordingly, one of ordinary skill would not be motivated to administer BoNT-E after the patient has developed antibodies against BoNT-A, because the antibodies against BoNT-A would cross react against BoNT-E. Thus, the prior art teaches away from administering BoNT-E after the patient has developed antibodies against BoNT-A.

By contrast, one of ordinary skill would be motivated to administer BoNT-F after the patient has developed antibodies against BoNT-A. For example, BoNT-F cleaves VAMP, not SNAP-25. Accordingly, one of ordinary skill would expect the two enzymes to be physically different. As BoNT-A is physically different from BoNT-F, one of ordinary skill would expect that antibodies against BoNT-A would not cross react with BoNT-F. Thus, one of ordinary skill would use BoNT-F after the patient has developed antibodies against BoNT-A.

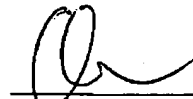
In summary, Applicant has surprisingly found that BoNT-E can effectively treat a dystonia in a patient after the patient develops antibodies against BoNT-A.

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In view of the foregoing, Applicant submits that the pending claims are in condition for allowance, and an early Office Action to that effect is earnestly solicited.

Respectfully submitted,



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# Communication

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## Identification of the Nerve Terminal Targets of Botulinum Neurotoxin Serotypes A, D, and E\*

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Botulinum neurotoxins are metalloproteins with one zinc atom bound to the zinc binding motif of zinc endopeptidases. Here we show that botulinum neurotoxin serotypes A, D, and E are zinc endoproteases specific for components of the synaptic vesicle docking and fusion complex. Serotypes A and E cleave SNAP-25, a 25-kDa protein of the synaptic terminal, while serotype D is specific for VAMP/synaptobrevin, a membrane protein of synaptic vesicles. Both rat brain VAMP isoforms are cleaved at a single Lys-Leu peptide bond. The proteolytic activity of these neurotoxins is inhibited by EDTA and captopril.

Botulinum neurotoxins (BoNTs)<sup>1</sup> penetrate motor neurons at the neuromuscular junction and block acetylcholine release, thus causing the flaccid paralysis of botulism (1). BoNTs are produced by *Clostridia* in seven different serotypes: A, B, C, D, E, F, and G. BoNT A and E are involved in human botulism, while serotype D is mainly responsible for animal botulism (2). BoNTs are increasingly used as therapeutic agents for several muscular dystonias (3).

BoNTs are composed of two disulfide-linked polypeptide chains. They bind to the presynaptic membrane via the heavy chain (H, 100 kDa) and enter the neuron cytosol, where the light chain (L, 50 kDa) blocks neuroexocytosis (1, 2, 4, 5).

Recently, we found that BoNT A, B, E, and F, as well as

tetanus neurotoxin (TeTx), contain one atom of zinc bound to the zinc binding motif of zinc endopeptidases, located in the middle of the L chains (6–10). The same zinc binding motif is also present in BoNT/D (11).

VAMP/synaptobrevin (VAMP), a highly conserved protein of synaptic vesicles (12–15), is the specific target of the proteolytic activity of TeTx, BoNT B, and BoNT F (6, 8, 16, 17). Of the two VAMP isoforms present in the rat nervous tissue (-1 and -2) (13), only VAMP-2 was cut by TeTx and BoNT B, while BoNT F cleaved both isoforms (8, 16). The cleavage by clostridial neurotoxins indicated that VAMP plays a key role in neuroexocytosis. Indeed, VAMP was recently implicated in docking and fusion of synaptic vesicles with the presynaptic membrane (18).

Here we show that BoNT serotypes A, D, and E are also zinc-dependent endoproteases. BoNT D acts specifically on VAMP, while BoNT A and E cleave a synaptosomal-associated protein of 25 kDa (SNAP-25) (19), another protein component of the putative vesicle docking and fusion apparatus (18).

### MATERIALS AND METHODS

**Proteins and Chemicals.**—TeTx and BoNT A, B, and E were obtained as described (6–8). BoNT D (Wako, Germany) was purified by ion exchange chromatography on a Mono Q PC 1.6/5 column (Pharmacia LKB Biotechnology Inc.). NSF,  $\alpha$ -SNAP,  $\beta$ -SNAP, and  $\gamma$ -SNAP were kindly provided by Drs. J. E. Rothman and T. Sollner, Sloan-Kettering Institute (New York). Rabbit polyclonal antisera against rat SNAP-25 and a mixture of rat brain VAMPs were prepared as reported (19, 20). A mouse monoclonal antibody against rat retina synaptophysin was purchased from Sigma. Peptide VLERDQKLSELD, synthesized and purified as before (8), and BoNT/F were a kind gift of Dr. C. C. Shone (PHLS, Porton Down, U.K.). Captopril ([2S]-1-[3-mercapto-2-methyl-propionyl]-1-proline) was from Squibb (Italy).

**Proteolytic Activity of Botulinum Neurotoxins on Synaptosomes, Synaptic Vesicles, and Synaptosomal Membranes.**—100  $\mu$ g of synaptosomes, isolated from rat brain cortex (21), were incubated with the different BoNT serotypes (100 nM) in HBM buffer (21), with or without 1% 1-O-n-octyl- $\beta$ -D-glucopyranoside, for 2 h at 37 °C. Synaptosomal membranes (LP1 fraction) and small synaptic vesicles (SSV) were isolated from rat cerebral cortex as described by Huttner *et al.* (22). 50  $\mu$ g of LP1 in 30  $\mu$ l of 6 mM HEPES-Na, 0.3 M glycine, 0.3 M NaCl, 0.02% NaN<sub>3</sub>, 1% 1-O-n-octyl- $\beta$ -D-glucopyranoside, pH 7.4, were treated with the various toxins (100 nM), previously reduced with 10 mM dithiothreitol for 30 min at 37 °C, and incubated for 2 h at 37 °C. 45  $\mu$ g of SSV in 30  $\mu$ l of the same buffer were treated with BoNT D (15 nM), reduced as above. In some samples EDTA (10 mM) or captopril (1 mM) or peptide VLERDQKLSELD (1 mM) was present. At the end of the incubation, samples were solubilized in 8% SDS, 10 mM Tris-Ac, pH 8.2, 0.1 mM EDTA and boiled for 2 min. Some SSV samples, after incubation with BoNT D, were diluted with 5 mM HEPES-Na, 10 mM EDTA to a final volume of 100  $\mu$ l and centrifuged on 10 mM HEPES-Na, pH 7.4, 120 mM NaCl for 60 min at 350,000  $\times$  g in a Beckman TL-100 centrifuge. Pellets were solubilized as above. Supernatants were treated with trichloroacetic acid (final concentration, 7%), centrifuged, and pellets processed as above. Samples were electrophoresed and stained with Coomassie Blue or with silver staining as before (8). Other samples, after electrophoresis, were transferred on nitrocellulose membranes for 3 h at 400 mA and treated with anti-VAMP (1:400 dilution) or with anti-SNAP-25 (1:2,500) or with anti-synaptophysin (1:10,000) antibodies for 16 h at 4 °C. The primary antibodies were detected with alkaline phosphatase or peroxidase-conjugated anti-IgG antibodies (2 h at 20 °C). Staining was performed either with the ECL chemiluminescence reagent (Amersham, UK) or with bromochloroindolyl phosphate/nitroblue tetrazolium. Immunoblots were quantitated by scanning with a dual wavelength Shimadzu CS-630 densitometer.

**Protein Sequencing.**—For sequence analysis, VAMP fragments were electroeluted from polyacrylamide gels, applied to ProSpin tubes, and sequenced in a pulsed-liquid Applied Biosystems model 477A Protein Sequencer.

\* This work was supported by grants from Telethon-Italia (to C. M. and F. B.), the Consiglio Nazionale delle Ricerche Target Project "Biotecnologie e Biostrumentazione" (to C. M.), MURST 60% (to F. B.), and by National Institutes of Health Grant NS17742 (to B. R. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> The abbreviations used are: BoNT, botulinum neurotoxin; TeTx, tetanus neurotoxin; SNAP-25, synaptosomal-associated protein of 25 kDa; NSF, N-ethylmaleimide-sensitive factor; SSV, small synaptic vesicles; VAMP, VAMP/synaptobrevin.

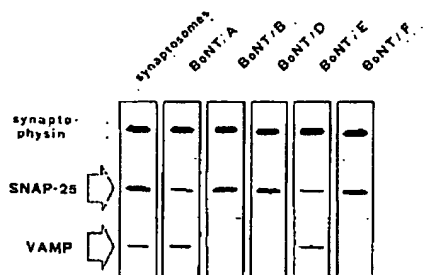


Fig. 1. Effect of BoNT A, B, D, E, and F on the level of synaptophysin, SNAP-25, and VAMP of rat brain synaptosomes. Rat brain synaptosomes were treated with either BoNT A, B, D, E, or F as detailed under "Materials and Methods," electrophoresed, and blotted onto nitrocellulose membranes. Different membranes were revealed with mouse anti-synaptophysin monoclonal IgG, rabbit anti-SNAP-25, and rabbit anti-VAMP antisera and visualized by chemiluminescence (5-s exposure).

## RESULTS AND DISCUSSION

TetTx and BoNT B and F are zinc endoproteases specific for VAMP/synaptobrevin, a protein localized on the membrane of synaptic vesicles (6, 8, 12, 13, 16). The other BoNT serotypes also contain the structural features of zinc endoproteases (7, 10), but their targets were not detected. Very recently, Sollner *et al.* (18) have shown that the interaction of synaptic vesicles with the target membrane is mediated by a multiprotein complex formed by VAMP/synaptobrevin, syntaxin (23), NSF (24), SNAP-25 (19), and two SNAPS (soluble NSF attachment proteins)  $\alpha/\beta$  and  $\gamma$  (25, 26). We have tested all these factors as likely candidates for the putative proteolytic activity of the available BoNTs of yet unknown target either with purified proteins or with different subcellular fractions from brain homogenate.

Rat brain synaptosomes were intoxicated with BoNT A, B, D, E, and F as reported (21), solubilized, subjected to SDS-polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membrane. Fig. 1 shows the result of the immunostaining with antisera against rat SNAP-25, VAMP, and synaptophysin. BoNT D has the same specificity of BoNT B and F, known to proteolyze specifically VAMP (9, 12). At variance, BoNT A and E cause a specific depletion of SNAP-25. None of the toxins was able to cleave NSF,  $\alpha$ -SNAP, or  $\gamma$ -SNAP (not shown). Though BoNT B, F, and D (see below) cleave VAMP at a single site, thus generating two fragments, no VAMP or SNAP-25 fragments were detected by immunoblotting. In the case of VAMP, this is due to a poor transfer of the fragments to the nitrocellulose paper (not shown). BoNT A and E caused an incomplete proteolysis of SNAP-25, a result attributable to incomplete accessibility of the substrate rather than to a slower rate of proteolysis with respect to serotype D. This interpretation of the results is supported by the data reported below.

To overcome the problem of substrate accessibility, rat brain synaptosomes were treated, before incubation with the BoNTs, with 1.0% 1-*O*- $\alpha$ -octyl- $\beta$ -D-glucopyranoside, a detergent that does not affect the activity of other clostridial neurotoxins such as TetTx and BoNT B and F.<sup>2</sup> Fig. 2 shows that, under these conditions, all the BoNT serotypes cause the nearly complete disappearance of their substrates within 2 h at 37 °C. Densitometric scanning of the immunoblots indicated that less than 20% of SNAP-25 and less than 5% of VAMP was left intact after incubation with the toxin.

Fig. 2 also shows that BoNT proteolysis of VAMP and SNAP-25 is completely inhibited by EDTA and captopril, indicating that BoNT A, D, and E are zinc-dependent endoprotein-

<sup>2</sup> O. Rossetto, G. Schiavo, and C. Montecucco, unpublished results.

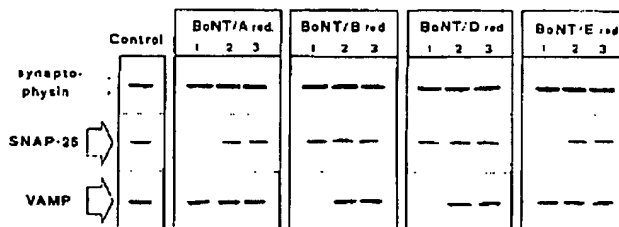


Fig. 2. Proteolytic activity of BoNT A, B, D, and E in permeabilized rat brain synaptosomes. 1, rat brain synaptosomes were treated with 1% octyl glucoside and then incubated with reduced BoNT A, B, D, or E, as described under "Materials and Methods." Same samples were treated with: 2, EDTA (final concentration 4 mM) or 3, captopril (final concentration, 1 mM). Specific synaptosomal proteins were detected as in Fig. 1.

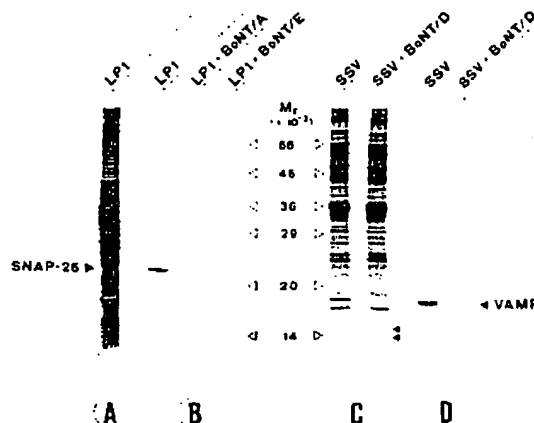


Fig. 3. Proteolytic activity of BoNT A, E, and D on synaptosomal membranes and on small synaptic vesicles. 50  $\mu$ g of SNAP-25-enriched synaptosomal membrane (LPI) was treated with reduced BoNT A and E (60 nM) for 2 h at 37 °C and electrophoresed in a 13–18% linear polyacrylamide gradient gel (8). A shows a silver stained control; neurotoxin-treated samples gave an identical protein profile (not shown). B, other samples were blotted on nitrocellulose membranes and stained by Western blotting by the anti-SNAP-25 antiserum and revealed by the alkaline phosphatase method. C, 35  $\mu$ g of SSV were incubated with reduced BoNT D (15 nM) for 2 h at 37 °C, electrophoresed, and stained as described (8). D shows the corresponding Western blot analysis with anti-VAMP antiserum revealed as in B.

ases. The inhibition by captopril is particularly interesting since this drug is widely used as an anti-hypertensive agent because it specifically inhibits the angiotensin-converting zinc endopeptidase (27). This opens the possibility of finding new inhibitors, highly specific for BoNTs, to be tested as therapeutic agents in the treatment of botulism.

To identify and isolate the fragments resulting from SNAP-25 proteolysis, we assayed a synaptosomal membrane preparation enriched in SNAP-25 (19). Fig. 3 shows that, in this subcellular fraction as well, BoNT A and E very effectively reduced the amount of SNAP-25 (panel B). However, this membrane preparation contains such a number of proteins migrating faster than SNAP-25 that the identification of SNAP-25 proteolytic fragments was not possible (panel A).

By contrast, the much simpler protein electrophoretic profile of SSV in the lower  $M_r$  region and the relative abundance of VAMP enabled us to identify the VAMP fragments induced by BoNT D. As shown in panel C of Fig. 3, BoNT D causes the disappearance of VAMP with the concomitant formation of two fragments of 9 and 8 kDa. This result is indicative of a single proteolytic cleavage site. No smaller peptides could be detected even in highly cross-linked polyacrylamide gels. Rat brain VAMP is completely converted by BoNT D in the 9- and 8-kDa

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## Botulinum Toxin A, D, and E Proteolytic Activity

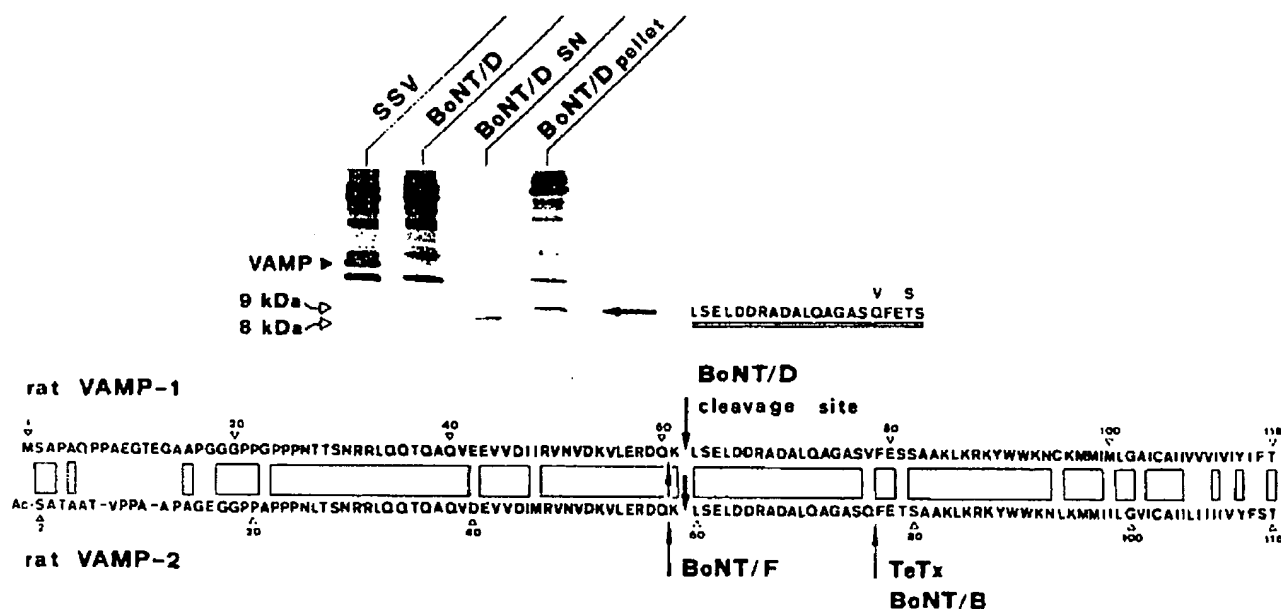


FIG. 4. Determination of the site of proteolytic cleavage of VAMP by BoNT D. The upper panel shows the Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis profile of small synaptic vesicles incubated with (BoNT D) or without (SSV) BoNT D as in the legend of Fig. 3. A BoNT D-treated SSV sample was ultracentrifuged, and the protein profiles of supernatant (SN) and pellet fractions are shown. The 8- and 9-kDa fragments, indicated by arrows, were electroeluted and sequenced. The amino terminus of the 8-kDa fragment was blocked, while the 9-kDa fragment gave the sequence reported on the right-hand side. The lower panel reports the sequences of rat brain VAMP-1 and -2 isoforms (13) and summarizes the sites of cleavage of botulinum neurotoxins serotypes B, D, and F and of tetanus toxin.

fragments, suggesting that both rat brain VAMP isoforms are sensitive to BoNT D.

VAMP is anchored to vesicles by a COOH-terminal hydrophobic tail, while the rest of the molecule is exposed to the cytosol. To separate and isolate the NH<sub>2</sub>- and COOH-terminal VAMP fragments, BoNT D-treated SSV were diluted in a saline solution and ultracentrifuged. Supernatant and pellet fractions were electrophoresed. Fig. 4 shows that the 8-kDa fragment is released into the medium and hence derives from the NH<sub>2</sub> terminus of VAMP, while the 9-kDa fragment remains attached to the vesicles and hence contains the COOH terminus. The two fragments were electroeluted and sequenced. No sequence was obtained for the supernatant VAMP fragment, as expected from the fact that its NH<sub>2</sub> terminus is blocked (18). At variance, sequencing of the pellet fragment gave the sequence LSELD-DRADALQAGASQ(V)FEST(S)TIS, which corresponds to rat brain VAMP-1 starting from Leu-62 and to VAMP-2 starting from Leu-60 (13), as schematically shown in Fig. 4. Cleavage of both rat brain VAMP isoforms is also demonstrated by the finding that at the 17th step both Gln and Val and at the 20th step both Ser and Thr were released. Neuronal rat VAMP-1 and -2 differ for these residues at these positions of their sequences. This identification of the cleavage site is confirmed by the fact that a peptide spanning the cleavage site, such as VLERDQKLSELD, inhibits the proteolytic activity of BoNT D (not shown).

Here we have provided evidence that BoNT A, E, and D are zinc endopeptidases. BoNT A and BoNT E cleave SNAP-25, while BoNT D acts on VAMP, the same substrate recognized by TeTx and BoNT B and F. However, BoNT D cleaves VAMP at a different site, as summarized in Fig. 4.

The fact that BoNT D acts on the same protein as BoNT B and F, while BoNT A and E recognize a different target, is in complete agreement with previous electrophysiological investigations, which have divided these neurotoxins into two groups: A-E and B-D-F-TeTx types (28, 29). These findings reemphasize the importance of VAMP in neuroexocytosis and highlight the

role of SNAP-25. This protein is localized at the synaptic terminal, and it is bound to the presynaptic membrane via palmitoyl chains (30). Its role in neuroexocytosis is still to be determined, but the present results, together with the recent reports that SNAP-25 is part of a putative vesicle docking and fusion apparatus (18) and that it is essential for axonal growth (31), provide functional evidence that SNAP-25 plays a central role in nerve terminal physiology.

It is noteworthy that the various clostridial neurotoxins, which have the common property of inducing a dramatic and sustained blockade of neurotransmitter release, do not attack a single site of the nerve terminal. Rather, the different toxins show multiple sites of proteolysis of different targets, involved in the same vesicle docking and fusion protein complex. However, the common zinc-dependent proteolytic mechanism of action of TeTx and of the various BoNT serotypes suggests the possibility of finding metalloprotease inhibitors useful in the treatment of tetanus and botulism patients.

**Acknowledgments**—We thank W. Topp for the preparation of BoNT A, B, and E neurotoxins, Drs. J. E. Rothman and T. Sollner for providing samples of NSF and SNAPs, and Dr. C. C. Shone for a peptide and BoNT F sample.

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